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Update on Molecular Epidemiology of H1, H5, and H7 Influenza Virus Infections in Poultry in North America

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SUMMARY. Avian influenza is endemic in wild birds in North America, and the virus routinely has been transmitted from this reservoir to poultry. Influenza, once introduced into poultry, can become endemic within the poultry population. It may be successfully eradicated by human intervention, or the virus may fail to successfully spread on its own. In the last 5 yr, influenza virus has been isolated from poultry in the United States on numerous occasions, and, with the use of molecular epidemiology, the relationships of these different viruses can be determined. There are 15 different hemagglutinin subtypes of avian influenza viruses, but infections with virus of H5 and H7 subtypes are of the most concern because of the potential for these viruses to mutate to the highly pathogenic form of the virus. Most of the influenza isolations in the United States have been associated with the live-bird markets (LBMs) in the Northeast. This has included primarily H7N2 influenza viruses, but also H7N3, H5N2, and other subtypes. Most of the H7N2 viruses were part of a single lineage that was first observed in 1994, but new introductions of H7N2 and H7N3 were also observed. The predominant H7N2 LBM lineage of virus spread to large commercial poultry operations on at least three occasions since 1997, with the largest outbreak occurring in Virginia in 2002. The H5N2 viruses in the LBMs included viruses from domestic ducks, gamebirds, and environmental samples. Some H5N2 viruses isolated in different years and in different locations had a high degree of sequence relatedness, although the reservoir source, if it is endemic, has not been identified. Finally, an H1N2 virus, associated with a drop in egg production, was isolated from turkeys in Missouri in 1999. This virus was a complex reassortant with swine, human, and avian influenza genes that was similar to recent swine isolates from the Midwest. Additional serologic evidence suggests that flocks in other states were infected with a H1N2 virus.

RESUMEN. Actualización sobre la epidemiología molecular de las infecciones por virus de influenza H1, H5 y H7, en la avicultura de Norte América.

La influenza aviar es endémica en aves salvajes en América del Norte y ha sido transmitida rutinariamente a las aves domésticas desde su reservorio natural. Una vez introducida en las aves domésticas, la influenza puede llegar a ser endémica dentro de la población avícola. La influenza aviar puede ser erradicada con éxito mediante la intervención humana o el virus puede dejar de difundirse por sí mismo. En los Estados Unidos durante los últimos cinco años, se ha aislado el virus de influenza en varias ocasiones a partir de aves domésticas. Mediante el uso de la epidemiología molecular se pueden determinar las relaciones existentes entre los diferentes virus. En los virus de influenza aviar existen 15 subtipos diferentes de hemoaglutinina, sin embargo, las infecciones con los subtipos H5 y H7 son las de mayor preocupación debido al potencial de estos virus para mutar a la forma de patogenicidad alta. La mayoría de los aislamientos del virus de influenza en el Noreste de los Estados Unidos han sido asociados con los mercados de aves vivas, incluyendo principalmente los virus de influenza H7N2 así como los virus H7N3, H5N2 y otros subtipos. La mayoría de los virus H7N2 fueron parte de un linaje observado por primera vez en

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1994, pero también se observaron nuevas introducciones de los virus H7N2 y H7N3. El linaje predominante del mercado de aves vivas H7N2 se ha difundido a operaciones avícolas comerciales grandes al menos en tres ocasiones desde 1997, con el mayor brote observado en el estado de Virginia en el año 2002. En el mercado de aves vivas, los virus H5N2 incluyeron virus de patos domésticos, aves de caza y muestras del medio ambiente. Algunos de los virus H5N2 aislados en diferentes años y en diferentes lugares tuvieron una alta relación en sus secuencias, aunque el reservorio, si es endémico, no ha sido identificado. Finalmente, se aisló un virus H1N2, asociado con una baja de postura, a partir de pavos en el estado de Missouri en 1999. Este virus fue producto de un reordenamiento complejo con los genes de virus de influenza de porcinos, humanos y aves, similar a los aislamientos obtenidos recientemente a partir de cerdos en el Medio Oeste. Evidencia serológica adicional sugiere que los lotes de aves en otros estados se infectaron con el virus H1N2.

Key words: avian influenza, molecular epidemiology, H5, H7, H1, live-bird markets, waterfowl, ducks, chickens

Abbreviations: aa = amino acid; AI = avian influenza; HA = hemagglutinin; HPAI = high-pathogenicity avian influenza; LBM = live bird market; LPAI = low-pathogenicity avian influenza; NA = neuraminidase; RT/PCR = reverse transcription/polymerase chain reaction

Type A influenza viruses are segmented, negative-sense RNA, enveloped viruses that can infect a wide variety of birds and mammals. However, the original reservoir of the influenza viruses is considered to be in wild waterfowl, gulls, and shorebirds (8,12). The virus demonstrates a high degree of genetic variation, particularly in the hemagglutinin and neuraminidase genes, with 15 different hemagglutinin and 9 neuraminidase subtypes being characterized from wild birds (13). Many of these hemagglutinin (HA) and neuraminidase (NA) subtypes have also been isolated from poultry, although chickens and turkeys are not considered a reservoir for avian influenza virus (14). Influenza is also common in some mammalian species, including swine, horses, and humans, but with only selected HA and NA subtypes, including H1N1 in swine, H3N8 in horses, and H1N1 and H3N2 in humans. Because of the propensity of influenza viruses to reassort and to cross species, viruses with new combinations of genes can and do occur. For example, recent outbreaks of influenza in swine in North America have included not only the classic H1N1 viruses, but also H3N2 and H1N2 viruses (7,19).

For poultry, primarily chickens and turkeys, influenza outbreaks are thought to first occur from the spread of influenza viruses from wild birds, and with high density confinement rearing methods, the virus has a unique chance to adapt to the new species (4). Influenza viruses can have up to 15 different HA and 9 different NA subtypes in wild birds, although they are not equally distributed within the wildlife population. In surveys of wild ducks in North America, for example, H3, H4, and H6 HA subtypes

represented over 50% of the isolations (11), and the H13 subtype is found almost exclusively in gulls (8). The H5 and H7 subtypes, which are uncommonly isolated from wild birds (11), often cause influenza outbreaks in chickens and turkeys in the United States (Table 1) (USAHA reports, various years). The reason for the apparent subtype discrepancy between the distribution of influenza in wild ducks and in poultry remains unknown.

Avian influenza can manifest itself with a variety of symptoms in chickens and turkeys, from a clinically unapparent infection to one with high mortality. The virus can generally be separated into viruses that cause primarily a mucosal or respiratory infection, referred to as low-pathogenicity avian influenza (LPAI), and a virus that replicates systemically, which causes high-pathogenicity avian influenza (HPAI). High-pathogenicity avian influenza viruses cause high morbidity and high mortality, and it is an Office Internationale des Epizooties List A agent. HPAI is considered an exotic disease in the United States, although LPAI can mutate to the highly pathogenic form of the virus. Historically only H5 and H7 influenza viruses are associated with the high-pathogenicity phenotype of avian influenza. Because of the heightened concern about H5 and H7 influenza viruses, representative isolates for these outbreaks were sequenced and compared to existing sequence databases to determine the relationships of each virus to others in the sequence database using phylogenetic analysis. This sequence analysis is also referred to as molecular epidemiology, and it provides the most precise way of determining the relationships of virus isolates to each other.

Table 1. Isolation of H5 and H7 influenza poultry in the U.S. since 1997.

Year	State ^a	Subtype	Species	Notes
1997–2002	NJ, NY	H7N2	Various poultry	LBMsb
1997	CT	H7N2	Chickens	LBMsb
1997–1998	PA	H7N2	Chickens	Commercial outbreak
1998	NJ, NY	H5N2	Pheasants	
1998	NY	H5N2	Environmental ^c	LBMsb
1998	MN	H5N2	Chukkars	
1998	DE, NY	H7N2	Chickens, quail	
1999	NY	H7N3	Chickens	LBMsb
2000	DE	H7N2	Environmental	
2000	NJ, NY	H5N2	Ducks, chickens	LBMsb
2001	NJ, NY	H5N2	Ducks, avian	LBMsb
2001	FL	H7N2	Chickens	LBMsb
2001	PA	H7N2	Ducks, chickens	
2001	PA	H7N2	Chickens	Small commercial outbreak
2001	NY	H5N2	Ducks	
2002	MA	H7N2	Guinea fowl	LBMsb
2002	VA, NC	H7N2	Turkeys, chicken, quail	Large commercial outbreak
2002	ME	H5N2	Ducks	
2002	PA	H7N2	Chickens	LBMsb

^aStandard 2-letter abbreviation for states in the U.S.
^bLBMS = sampling from live-bird markets.
^cEnvironmental = isolation of influenza virus only from environment swabs of LBMs or other poultry-associated facilities.

The live-bird markets in the Northeast have been the source of most of the avian influenza virus isolations in the United States in the past few years. Avian influenza was first detected in the live-bird market system in 1986 when H5N2 influenza viruses related to the 1983–84 Pennsylvania outbreak were isolated (17). Influenza viruses of many different subtypes have been isolated almost every year since then. The live-bird market (LBM) system in the Northeast United States currently consists of about 120 markets spread throughout the larger cities in New York, New Jersey, Pennsylvania, and the other New England states. Most of the markets are in and around New York City. The markets sell a variety of live birds including chickens, pheasant, quail, ducks, turkeys, and other avian species. Some markets may also sell mammals, including rabbits, sheep, and goats. The customer typically selects the bird, which is then slaughtered and dressed on the premises and sent home with the consumer. The markets are typically supplied with fresh birds daily or every few days through a complex network of wholesalers and dealers. Most of the birds are bought from the farmer by a wholesaler who then delivers the bird to the individual market, although birds may be purchased

from auctions or delivered to the market directly by the farmer. The markets are supplied with birds from many different states, including such faraway states as North Carolina, Ohio, and Maine, but most of the birds are supplied from Pennsylvania. Efforts have been ongoing to ensure that birds being sold to the markets are from avian influenza–free flocks through ongoing testing. However, since birds are not individually identified, it is likely that not all the birds are from avian influenza (AI) tested flocks.

This report examines recent influenza isolates from North America to help answer epidemiological questions about each outbreak. As mentioned previously, H5 and H7 isolates are of special concern and will be examined in detail. Also, a recent outbreak in turkeys of an H1N2 virus will also be discussed because of the unique history of the virus.

MATERIALS AND METHODS

Sequence and phylogenetic analysis. Influenza isolates sequenced for this study were either from the SEPRL repository, from the National Veterinary Services Laboratories, or from state veterinary medical diagnostic laboratories. The RNA was either extracted

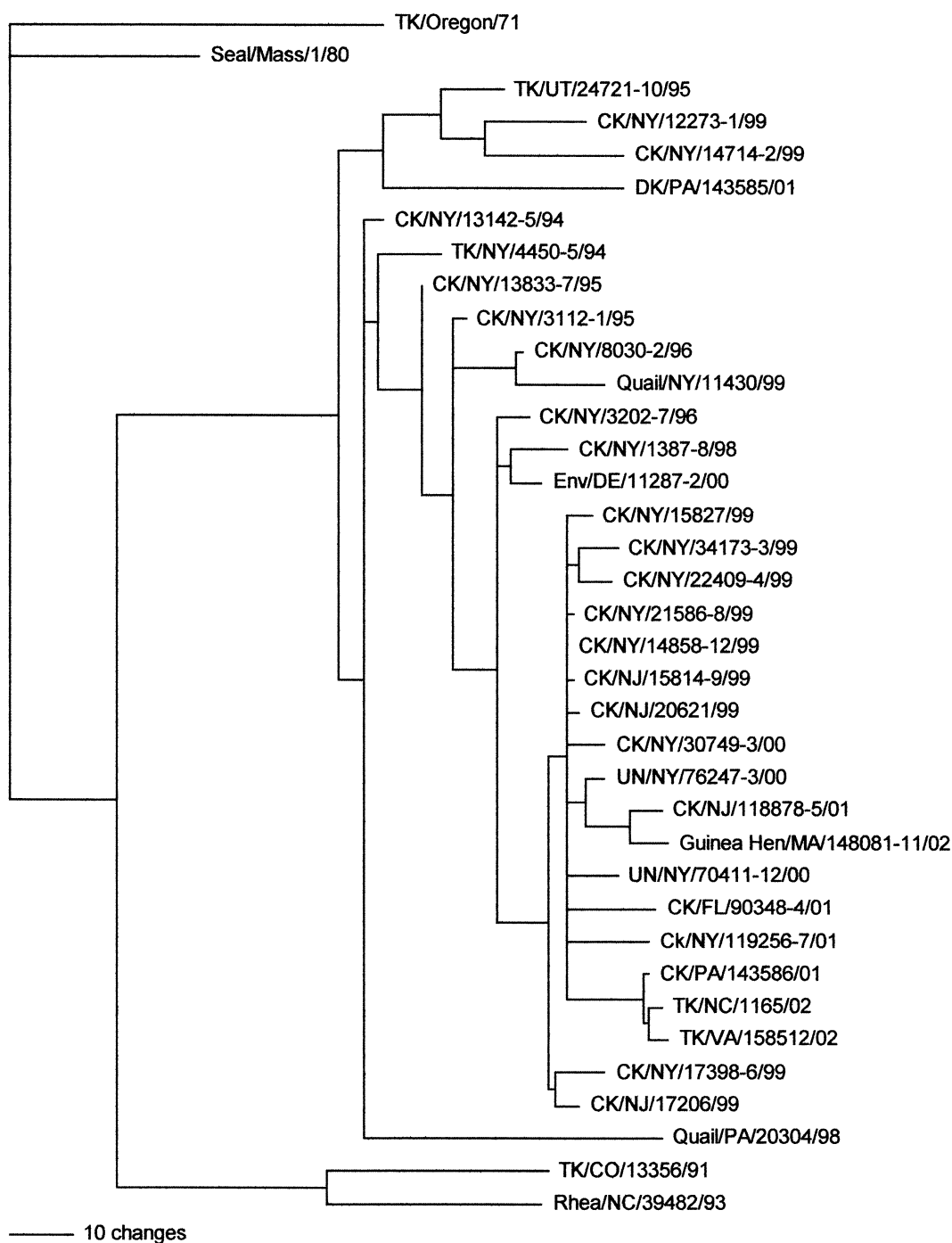


Fig. 1. Phylogenetic tree of the HA1 subunit of H7 subtype from representative avian influenza viruses from North America. The tree was generated with PAUP 4.0b4 computer program with bootstrap replication (100 bootstraps) and a heuristic search method. The tree is unrooted. Standard two-letter postal codes are used for states in the United States. Abbreviations used are TK = turkey, CK = chicken, DK = duck, Env = environmental, and UN = unknown avian.

from the original sample or the virus isolate was passaged an additional time in 10-day-old embryonated eggs before RNA extraction. Viral RNA was extracted using either Trizol LS (Invitrogen, Carlsbad, CA) or the Rneasy RNA extraction kit (Qiagen, Valencia, CA). The HA1 region of the hemagglutinin was sequenced for all isolates examined, and additional genes were sequenced from selected isolates. Each gene was reverse transcription/polymerase chain reaction (RT/PCR) amplified using gene specific positive sense primers using the OneStep RT/PCR amplification kit (Qiagen, Valencia, CA). The incubation period for the reverse transcription step was 48 C for 1 hr, then a denaturation step of 15 min at 94 C before the 30 cycles of denaturation at 94 C for 45 sec, annealing at 53 C for 15 sec and extension at 72 C for 1 min. The product was electrophoresed on a 1.5% agarose gel with ethidium bromide, and the DNA band of the expected size was excised from the gel. The DNA was extracted with the agarose gel DNA extraction kit (Roche Molecular Biochemicals, Indianapolis, IN). The PCR product was either quantified and directly sequenced or was cloned into the pAMP vector (Gibco, Gaithersburg, MD) and the plasmid was sequenced. Both the plasmid and PCR products were sequenced on an ABI3700 sequencer using the big dye terminator sequencing kits (Perkin Elmer Applied Biosystems, Foster City, CA). The sequencing information was compiled with the Seqman program (DNA-STAR, Madison, WI), and the nucleotide sequences were compared initially with Megalign program (DNASTAR, Madison, WI) using the Clustal V alignment algorithm. Phylogenetic comparisons of the aligned sequence for each gene were generated using the maximum parsimony method with 100 bootstrap replicates in a heuristic search using the PAUP 4.0b4 software (Sinauer Associates, Inc, Sunderland, MA).

RESULTS

In 1994, an H7N2 influenza virus was first isolated from the LBMs in New York and New Jersey in the northeast United States as part of a routine surveillance program. Several other influenza viral subtypes were also isolated, including H7N3 viruses from New Jersey (15). Since these original isolations, H7N2 influenza has been isolated every year from the LBMs. Other influenza subtypes have also been isolated, but most other subtypes were transient and were rarely isolated in consecutive years. A representative selection of H7 influenza viruses from the LBMs and other H7 influenza viruses from around the United States were sequenced and compared by phylogenetic analysis of the HA1 subunit of the hemagglutinin gene (Fig. 1). Most of the isolates from the LBM were part of the same lineage of virus as the index case in 1994. In 1999, several H7N3

viruses were isolated that had distinctly different HA1 genes from the primary H7N2 LBM lineage. These viruses also had an HA cleavage site consistent with a recent introduction from wild birds. The H7N3 viruses did not appear to persist in the LBM, since no further H7N3 isolations were made after 1999. One H7N2 virus isolated from quail from Pennsylvania in 1998 was phylogenetically different from the H7N2 LBM lineage, and it had an unusual HA cleavage site with an additional basic amino acid at the -5 position.

The H7N2 LBM lineage of viruses has previously been shown to have a high rate evolution with many progressive mutations occurring in the HA1 gene (15). These progressive amino acid changes occurred throughout the HA1 gene segment, but the changes that occurred at the HA cleavage site will be highlighted. In 1994, the first H7N2 viruses isolated had two basic amino acids at the cleavage site, including an arginine (R) at the -1 position and a lysine (K) at the -3 position. In 1995, an amino acid (aa) substitution occurred at the -2 position with a substitution of proline for threonine, and the variants with this sequence became the predominant isolates in the LBMs. In 1998, a second change occurred at the HA cleavage site with the additional basic aa lysine being substituted for asparagine at the -5 position, and this variant became the predominant isolate in the LBMs. In 2002 a third change occurred again at the -2 position with the substitution of the basic aa lysine for proline. It is unclear whether this new variant, which has four basic aa near the HA cleavage site, will become the predominant isolate in the market. One additional unique feature of the H7N2 LBM lineage was an eight aa deletion in the HA1 gene that was first observed in 1996. This variant has become the predominant lineage in the LBMs. All the H7N2 viruses that have been pathotyped by chicken inoculation studies have been classified as low pathogenicity.

The H7N2 LBM lineage viruses have been shown to have spread to large commercial poultry operations on at least three occasions. The first outbreak occurred in Pennsylvania in 1997-98 and was previously reported by Ziegler *et al.* (20). The second outbreak occurred in Pennsylvania in December 2001 and involved nine different farms. A summary of this outbreak is being presented as a separate paper at this symposium. The most recent outbreak is ongoing in Virginia, and further details are also being presented in a separate paper. The sequence analyses of the H7N2 virus from all three outbreaks demonstrate that the viruses were similar to the

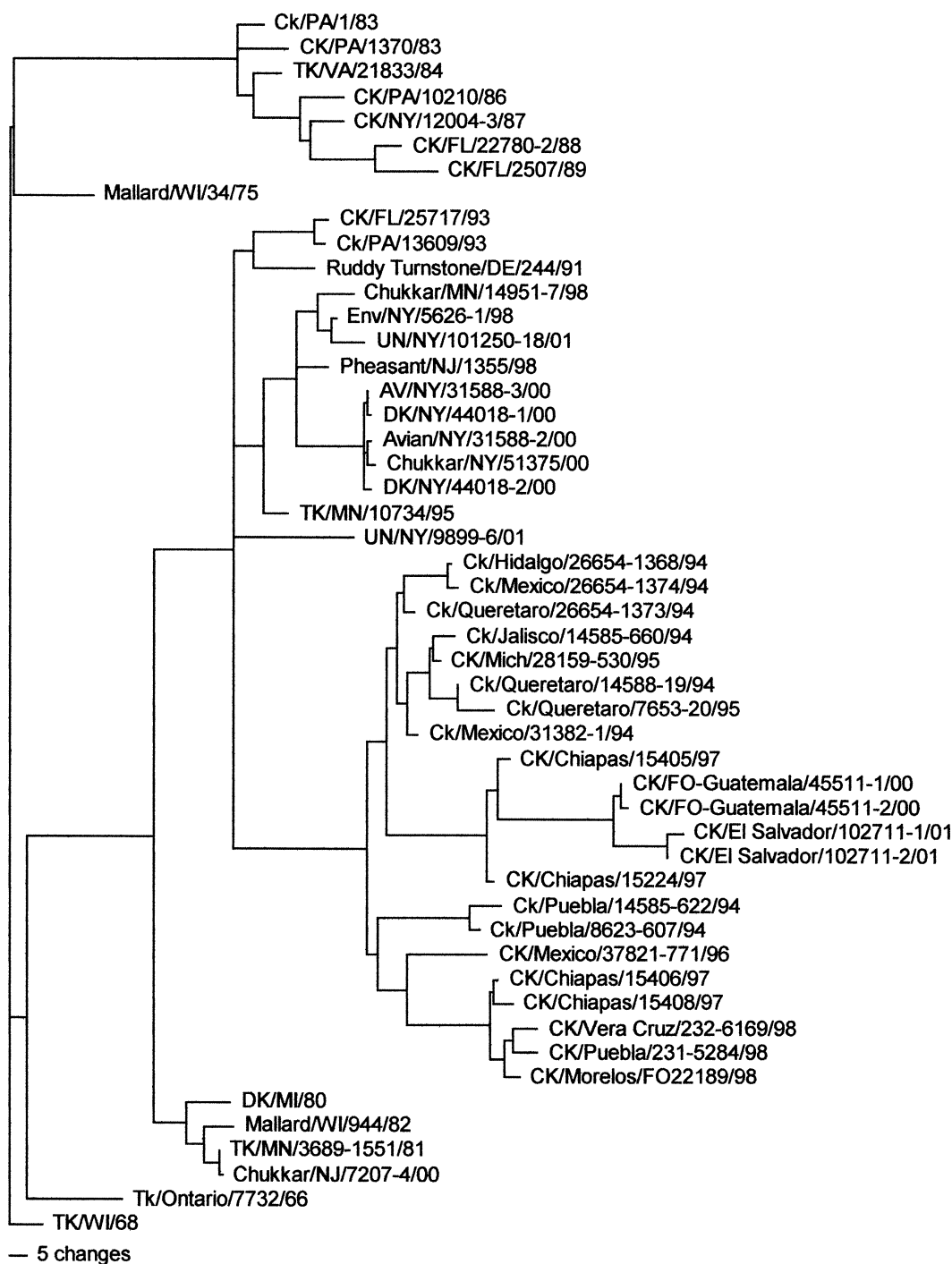


Fig. 2. Phylogenetic tree of the HA1 subunit of H5 subtype from representative avian influenza viruses from North America. The tree was generated with PAUP 4.0b4 computer program with bootstrap replication (100 bootstraps) and a heuristic search method. The tree is rooted to A/TK/WI/68. Standard two-letter postal codes are used for states in the United States. Abbreviations used are TK = turkey, CK = chicken, DK = duck, Env = environmental, and UN = unknown avian.

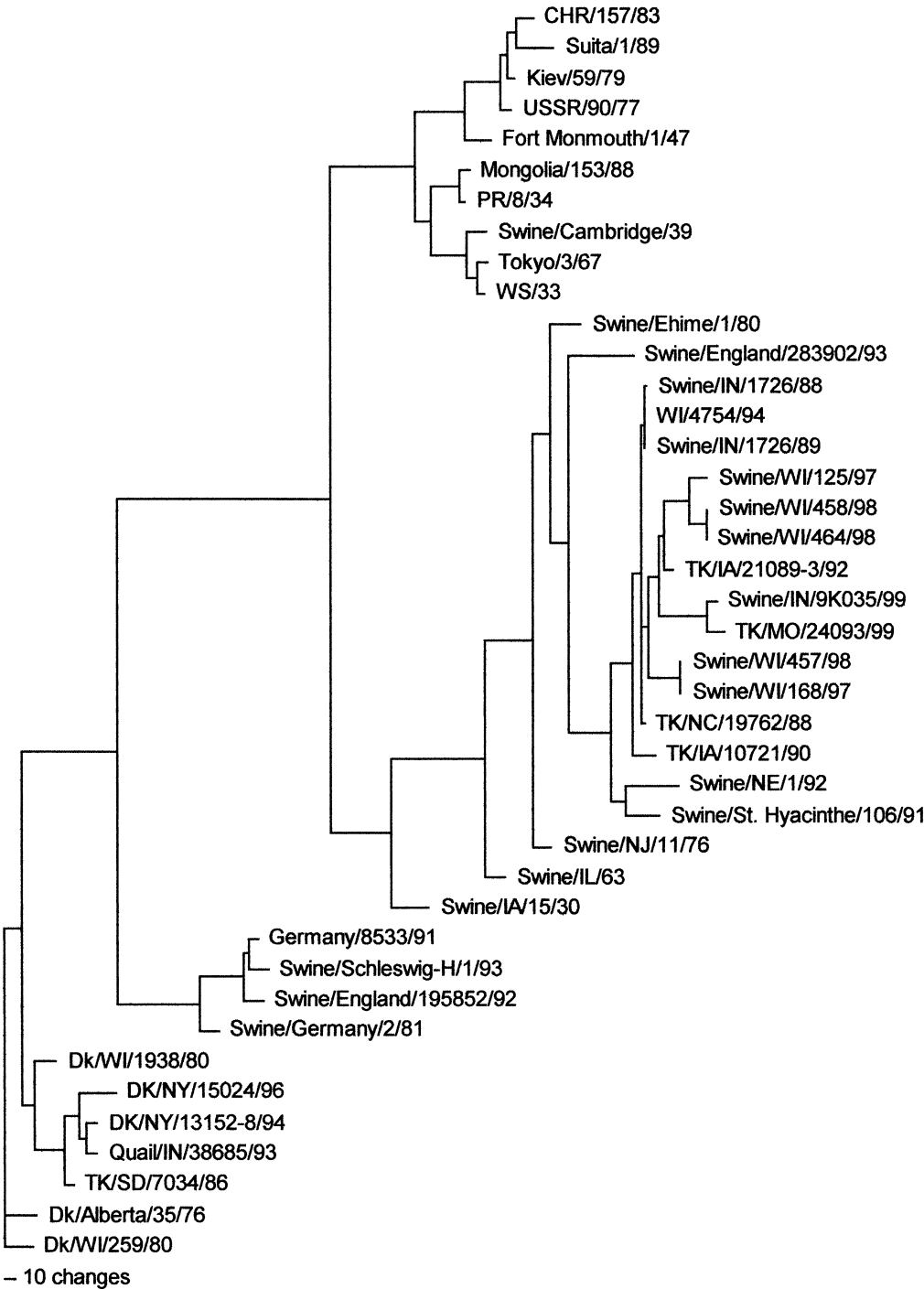


Fig. 3. Phylogenetic tree of the hemagglutinin subtype 1 nucleotide sequence, which includes A/TK/MO/24093/99 and representative type A human, swine, and avian influenza gene sequences. The tree was generated with PAUP 4.0b4 computer program with bootstrap replication (100 bootstraps) and a heuristic search method. The tree is rooted to DK/WI/259/80. Standard two-letter postal codes are used for states in the United States. Abbreviations used are TK = turkey, CK = chicken, and DK = duck. For isolates without a species, it is assumed to be from a human.

viruses circulating in the LBMs during the same time period (Fig. 1). The H7N2 LBM lineage of virus has also been isolated from birds in the LBM in Miami during 2001.

Fewer isolations of H5 subtype influenza viruses have been reported than H7 isolations, but again the Northeastern U.S. LBMs have been the source of several isolates. In the past 5 yr, H5N2 isolates have been made from LBMs including environmental samples in 1999 and from ducks in 2002. Other isolations have been made from pheasant, quail, and ducks as part of the surveillance programs required to sell birds to the LBMs. Many of these isolates seem to cluster on the phylogenetic analysis (Fig. 2), but it is not clear from detailed sequence analysis whether the isolates represent separate introductions from wild birds or an endemic infection in poultry, possibly in domestic ducks.

The H5N2 outbreak in Mexico that started in 1993 continues in Mexico today, although the isolates from 1998–02 have not been made available for sequence analysis. However, the Mexican H5N2 lineage has spread to two other Central American countries, with isolations from Guatemala in 2000 and El Salvador in 2001. The Mexican H5N2 lineage, although believed to be from a single introduction of virus, has resulted in several distinct lineages of virus in poultry populations. The Guatemala and El Salvador viruses appear to have come from the Chiapas lineage. The state of Chiapas is the southernmost state in Mexico and borders El Salvador.

In 1999 a turkey breeder flock in production from Missouri suffered a greater than 40% drop in egg production over a 9-day period. During the diagnostic evaluation of the flock, antibody to influenza was detected by the agar gel immunodiffusion test. Clinical samples of birds were submitted for virus isolation, and a H1N2 influenza virus was isolated (A/Turkey/MO/24093/99)(18). Although H1N1 infections in breeder turkeys have been reported, this was the first case of H1N2 reported from turkeys in the United States.

The isolate was further evaluated by sequencing at least part of all eight gene segments, and the sequence was compared phylogenetically with the influenza sequence database. The virus was a complex reassortant virus with gene segments of swine, human, and avian influenza lineage origin (18). The TK/MO/24093/99 isolate had hemagglutinin (Fig. 3), non-structural, matrix, and nucleoprotein genes that clustered with North American classic swine influenza viral genes. The neuraminidase and poly-

merase basic protein 1 (PB1) genes clustered with human influenza viruses, and the polymerase acid protein (PA) and polymerase basic protein 2 (PB2) genes clustered with North American avian influenza viral genes. This reassortant virus was also very similar to a 1999 swine influenza virus from Indiana (6).

Two additional turkey flocks from Missouri had evidence of H1N2 influenza infections after the index case, including one flock with a large drop in egg production. None of the flocks had been vaccinated with the commercially available H1N1 killed influenza vaccine. After the outbreak in Missouri an autogenous killed H1N2 vaccine was used to vaccinate other flocks within the affected company (18).

Since the original isolation of H1N2 from turkeys, no new H1N2 viruses have been isolated from turkeys in the United States, but H1N2 isolates have been isolated from swine at low levels (1). There has also been serologic evidence in the last 2 yr of H1N2 infections turkeys in Ohio and Illinois (10). It appears from the swine surveillance data and the serologic evidence in poultry that H1N2 reassortant influenza viruses are continuing to cause disease outbreaks in the United States.

DISCUSSION

The LBMs in the Northeast remain the biggest concern for the presence of avian influenza in the United States. The market system has had three different H5 or H7 lineages endemic since 1986, including an H5N2 virus related to the Pennsylvania HPAI in 1986–89 (17), a separate H5N2 virus in 1993 (9), and finally the H7N2 virus from 1994 to the present. Many other subtypes of avian influenza have also been isolated from the LBMs during this period of time, but these viruses do not seem to persist. Until just the past few years, no testing of birds for avian influenza was required before selling birds to the live-bird markets, so the requirement for pretesting of birds for AI is a major improvement to try to prevent the introduction of AI. However, by its design of using primarily serologic tests for AI, this system cannot prevent the introduction of all AI viruses. The LBM system, because of how it is allowed to operate, has several high-risk features for the introduction and perpetuation of avian influenza viruses. This includes the mixing of many different species of birds, including ducks. Domestic ducks are considered a risk factor because they often are in contact with wild birds and once infected are usually subclinical carriers of the virus. For these reasons, the LBM system in Hong

Kong segregates waterfowl, including ducks and geese, from other birds to reduce the transmission of influenza within the market system (3). The other major risk factors for the perpetuation of influenza in the U.S. markets are the introduction of influenza-naïve birds on an almost daily basis and the fact that most markets are seldom completely empty of birds. This provides a continual source of susceptible birds that can be infected from virus shed from residual birds or by virus left in the environment.

Influenza in the LBM system has also shown itself to be a major risk of infection for the large commercial poultry industry. Molecular epidemiological links between the LBMs and commercial poultry have occurred with H5N2 viruses in 1985–86 (17), 1993 (9), and with H7N2 in 1997–98 (20), 2001, and 2002. Considerable efforts are continuing on the part of industry and state and federal governments to control influenza in the LBM system, but currently the efforts have been unsuccessful.

The introduction of avian influenza into commercial poultry continues to be a problem, but it appears infrequently and is unpredictable. The occurrences of avian influenza in turkeys in Minnesota that has occurred every fall has been greatly reduced (4). This drop in the number of infected flocks likely relates to the change in management where most turkeys now are reared completely in confinement and do not have the exposure to migratory waterfowl that they once did. The sporadic introduction of avian influenza to individual farms will likely continue for the foreseeable future, but with adequate biosecurity procedures the virus should not spread to other farms. However, the rapid spread of AI in the recent outbreak in Virginia has suggests that the biosecurity on many farms is inadequate.

The continued presence of H5N2 in Mexico has also been a risk factor for its neighbors. The control strategy for influenza in Mexico, which has included the use of vaccination, has not been able to eliminate H5N2 avian influenza from the country of disease after 9 yr of use. The continued presence of H5N2 in Mexico was the likely source of infection for flocks in Guatemala and El Salvador. It is unclear why the H5N2 virus lineage that resulted in several outbreaks of HPAI in Mexico in 1994–95 has remained of low pathogenicity.

One additional way for influenza to be introduced into a naïve flock is by contact with a mammalian-adapted influenza virus. The mammalian influenza virus that has been most commonly associated with poultry infections in the past is classical H1N1 swine influenza virus. Several reports have described

clinical disease in turkey breeders associated with H1N1 swine influenza viruses in the United States (2,5). The threat of swine influenza viruses causing outbreaks in turkeys has resulted in the common use of killed H1N1 influenza vaccines in turkey breeders. The current outbreak of H1N2 in turkeys in Missouri was unusual because this was the first reported case of H1N2 infection in turkeys in the United States. The sequence analysis of the virus also demonstrated that the virus was a complex reassortant of human, swine, and avian influenza viral genes. A similar virus had also been isolated from swine in Indiana (6), and it is likely that the turkeys were again infected with a swine-origin influenza virus. The flocks in Missouri that were infected with the H1N2 viruses had not previously been vaccinated against the H1N1 influenza, and because influenza vaccine protection primarily comes from antibodies to the hemagglutinin protein (16), vaccination would likely have protected this flock.

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